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(54) Title: ENGINEERED SECRETED ALKALINE PHOSPHATASE (SEAP) REPORTER GENES AND POLYPEPTIDES

(57) Abstract: The invention provides a novel reporter gene (mSEAP) capable of being expressed in a mammal for extended periods of time. Nucleic acids encoding the reporter gene, cells and vectors comprising the nucleic acids, and methods of using the reporter gene to identify expression vectors and screen for drug compounds in an animal are also disclosed.

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INTERNATIONAL SEARCH REPORT

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A. CLASSIFICATION OF SUBJECT MATTER
 IPC 7 C12Q1/68 C12N9/16

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 IPC 7 C12Q C12N

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C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 327 960 A (HOFFMANN LA ROCHE) 16 August 1989 (1989-08-16) see whole doc. esp. .5, 1.20-55, p.11, exp.5,	1-35
X	--- BETTAN M. ET AL.,: "secreted human placental alkaline phosphatase as a reporter gene or in vivo gene transfer" ANAL. BIOCHEMISTRY, vol. 271, - 1999 pages 187-189, XP002191938 the whole document --- -/--	1-35, 37-65

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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- *A* document defining the general state of the art which is not considered to be of particular relevance
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- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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Date of the actual completion of the international search

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>GREGORY B ET AL: "A FAST AND SENSITIVE COLORIMETRIC ASSAY FOR IL-6 IN HEPATOMA CELLS BASED ON THE PRODUCTION OF A SECRETED FORM OF ALKALINE PHOSPHATASE (SEAP)9"</p> <p>JOURNAL OF IMMUNOLOGICAL METHODS, ELSEVIER SCIENCE PUBLISHERS B.V.,AMSTERDAM, NL, vol. 170, 29 March 1994 (1994-03-29), pages 47-56, XP000601567 ISSN: 0022-1759 the whole document</p>	1-35, 37-65
A	<p>BARTLETT R J ET AL: "LONG-TERM EXPRESSION OF A FLUORESCENT REPORTER GENE VIA DIRECT INJECTION OF PLASMID VECTOR INTO MOUSE SKELETAL MUSCLE: COMPARISON OF HUMAN CREATINE KINASE AND CMV PROMOTER EXPRESSION LEVELS IN VIVO"</p> <p>CELL TRANSPLANTATION, ELSEVIER SCIENCE, US, vol. 5, no. 3, 1996, pages 411-419, XP002920729 ISSN: 0963-6897 see whole do. esp.p.414, discuss. and fig.4</p>	1-35, 37-65
A	<p>MANES T ET AL: "GENOMIC STRUCTURE AND COMPARISON OF MOUSE TISSUE-SPECIFIC ALKALINE PHOSPHATASE GENES"</p> <p>GENOMICS, ACADEMIC PRESS, SAN DIEGO, US, vol. 8, 1990, pages 541-554, XP001062172 ISSN: 0888-7543</p>	
P,X	<p>WANG M. ET AL.: "MUSEAP, a novel reporter gene for the study of long-term gene expression in immunocompetent mice" GENE, vol. 279, 14 November 2001 (2001-11-14), pages 99-108, XP004324228 the whole document</p>	1-35, 37-65

INTERNATIONAL SEARCH REPORT

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PCT/EP 02/06930

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 36
because they relate to subject matter not required to be searched by this Authority, namely:
The subject-matter of claim 36 is considered by this ISA as not patentable,
Rule 39 PCT
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such
an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all
searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment
of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report
covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is
restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/EP 02/06930

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
EP 0327960	A	16-08-1989	EP JP	0327960 A1 2005862 A	16-08-1989 10-01-1990
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ENGINEERED SECRETED ALKALINE PHOSPHATASE (SEAP)
REPORTER GENES AND POLYPEPTIDES

5 The invention relates to nucleic acids that encode reporter genes capable of being used in long term expression studies and vectors comprising them. Methods for producing vectors for long term expression and the expression systems that incorporate these reporter genes and other transgenes are also included in the invention, as well as the recombinant polypeptides encoded by the reporter genes.

10 Many gene transfer vectors and systems are intended to express genes for extended periods of time, i.e. over weeks or months. This is especially the case where the transgene encodes a functional protein or therapeutic protein. Methods to evaluate the persistence of expression using these gene transfer vectors usually employ detectable reporter genes. However, the protein products of currently used reporter
15 genes lack one or more of several characteristics, making them inappropriate for studying long term expression in an animal. This invention provides new and useful reporter genes, nucleic acids, expression vectors, polypeptides, and methods of expressing transgenes that are particularly suited for long term expression. Compared to the prior reporter genes, which may express detectable protein up to 20
20 days or so, the reporter genes of the invention express detectable protein levels for more than about a month, or to at least about 9 months. The expression levels are also stable over this period. Furthermore, the proteins from reporter genes of the invention can be detected in a number of cell types and tissues, where detection using other reporter genes has proven to be difficult.

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Reporter genes have been used to analyze the expression of transgenes from various vectors. Reporter genes that have been used in animal models encode exogenous cytoplasmic or secreted proteins, such as bacterial β -galactosidase, insect luciferase, human growth hormone, human erythropoietin, and human secreted alkaline

phosphatase (SEAP). These reporter genes often are used for transient expression studies or tissue-specific expression studies. However, the proteins they encode are typically immunogenic. They can also elicit a cytotoxic T-lymphocyte response or a neutralizing antibody response that suppresses detection, leading to inaccurate reporter gene expression data (see Tripathy *et al.*, Nature Medicine 2:545-50 (1996); and Yang *et al.*, Gene Therapy 3:137-44 (1996)).

From these deficiencies alone, one can conclude that the reporter genes currently used were not really designed for and have not been shown to be amenable to longer term expression studies. When longer term expression data is needed, a reporter gene that continues to express, can be detected, avoids the animal's immune response mechanisms, and does not alter cell physiology is important. To date, such a reporter gene has not been adequately provided. In fact, experimental systems have been modified to accommodate the existing reporter genes by, for example, using immuno-deficient animals.

One reporter gene, the SEAP gene noted above, is derived from the native human placental alkaline phosphatase (hPLAP). The amino acid sequence typically used for its reporter gene function differs from the native gene by a deletion of C-terminal residues, which converts the membrane-bound protein into a secreted protein (Berger, *et al.*, Gene 66, 1-10 (1988)). While the SEAP reporter gene has been used in a number of expression systems, the selection of alkaline phosphatase-derived reporter genes often results in detection problems, especially when long term expression levels are analyzed. For example, background alkaline phosphatase activity can be present in mammalian tissue. Also, inhibitors of alkaline phosphatase action can be present in certain mammalian tissues. These and other shortcomings of the SEAP gene and other alkaline phosphatase-derived reporter genes have yet to be overcome.

The invention encompasses alkaline phosphatase (AP) reporter genes and nucleic acid sequences encoding a mammalian alkaline phosphatase activity. The alkaline phosphatase activity is capable of being detected for at least about one month after being inserted into a cell as a transgene. Preferably, expressing the alkaline phosphatase-encoding sequences of the invention in a cell results in alkaline phosphatase activity that can be detected for more than about 40 days, or more than about 60 days, or more than about 90 days, or more than about 120 days, or more than about 180 days, or more than about 270 days. Typically, but not necessarily, the alkaline phosphatase activity is detected by measuring the protein levels or AP activity of a serum or tissue sample from a mammal containing the cell with the transgene, or measuring the media from a cultured cell containing the transgene. Other methods of detection can be used, including immunochemical assays.

In a preferred embodiment, the mammalian alkaline phosphatase activity consists of, consists essentially of, or comprises a polypeptide amino acid sequence of SEQ ID NO: 1 or SEQ ID NO: 2. A polypeptide or protein consisting essentially of the amino acid sequence of SEQ ID NO: 1 or SEQ ID NO: 2 will contain the amino acid sequence of SEQ ID NO: 1 or SEQ ID NO: 2 and one or more amino acid differences in the sequence that do not change the basic and novel characteristics of the protein. As described, some of the basic and novel characteristics of the mSEAP polypeptide of SEQ ID NO: 2 or the AP activity of SEQ ID NO: 1 are the ability to be expressed and detected in long term expression studies (here long term can mean one of over 30 days, 40 days, 60 days, 90 days, 180 days, or 270 days), the ability to be detected long term in immuno-competent mammals, and/or the ability to be expressed long term at stable levels (levels that do not change more than about 5-fold or more than about 10-fold). None of the prior alkaline phosphatase, embryonic alkaline phosphatase, or secreted embryonic alkaline phosphatase proteins or polypeptides possess even one of these novel characteristics. Any isolated or purified cDNA or nucleic acid that encodes a polypeptide of SEQ ID NO: 1 or SEQ

ID NO: 2 is included in the invention. Also, the polypeptide of SEQ ID NO: 1 can be joined with an appropriate mammalian or murine signal sequence so the AP activity is secreted from the cell. Any of a variety of signal sequences can be used, including that of SEQ ID NO: 3, 9, 10, 11, or 12. Fusion proteins where the amino acid sequence of SEQ ID NO: 1 or 2 is used are also included in the invention provided they do not include a fusion generating or encoding the exact amino acid sequence of SEQ ID NO: 13. Furthermore, derivatives and mutants of SEQ ID NO: 1 or 2 are included in the invention and specific, non-limiting examples of the mutants are presented. One series of mutants includes polypeptides where about 1 to about 5 amino acids are deleted from the C-terminal end of SEQ ID NO: 1 or 2. One skilled in the art can use numerous methods to create other derivatives or mutations in SEQ ID NO: 1 or 2 and the degenerate variants encoding SEQ ID NO: 1 or 2 as well as the derivative polypeptides and mutant polypeptides themselves. The derivative polypeptides, and the nucleic acids encoding them, also possess or encode polypeptides that possess one or more of the novel long term expression characteristics listed above, but do not include a polypeptide or SEQ ID NO: 13.

In another aspect, the invention comprises methods for analyzing expression systems and vectors for use in gene transfer experiments and protocols. For example, a gene transfer vector that comprises a reporter gene or nucleic acid sequence of the invention can be introduced into an animal. By detecting reporter gene expression levels, the vector system components and methods used to introduce and prepare the vector for administration can be tested and/or optimized for long term use in expressing a transgene. The methods can be used to select a type of expression vector, specific regulatory or combinations of sequences used in expression vectors, and protocols for administering transgenes to animals. For example, the properties or desirability of selected gene expression regulatory elements (DNA or RNA sequences in *cis*, or protein, DNA, or RNA factors in *trans*) can be analyzed and/or optimized by using the nucleic acids and methods of the invention. Both the

selection and analysis processes and the vectors and compositions selected or that result from the processes are specifically included in this invention. In a preferred embodiment of this aspect, a vector is prepared and introduced into an animal or cell. The vector comprises a reporter gene or nucleic acid of the invention linked to an appropriate promoter or promoter/enhancer so that the reporter gene is expressed. The alkaline phosphatase expression levels are analyzed for at least about one month and optionally compared to controls, such as different vectors comprising the reporter gene. The results of the expression levels for the period of at least about one month are then used to select or confirm that a vector is appropriate or desirable for the administration of a particular transgene. The same methods can be used for periods of at least about 40 days, about 60 days, about 90 days, about 120 days, about 180 days, or about 270 days. The selected vector with particular transgene can then be used for other purposes where long term expression is desired, such as treatment for disease or physiological condition or production of a certain phenotype in an animal. Similarly, administration methods can be analyzed and compared, as for example intramuscular injection, intratumoral injection, intradermal injection, inhalation, or other routes of administration. The type of vector used can also be analyzed, such as naked plasmid, adenoviral vector, adeno-associated virus vector, retroviral vector, and lentiviral vector. The present invention is also directed to reporter vector containing the mSEAP sequence according to the present invention. Such reporter vector may be a prokaryotic plasmid such as, pBR322 or pUC plasmid containing a gene of resistance, such as, *inter alia*, ampicilline, tetracycline, or kanamycine gene, an origin of replication functional in eukaryotic cells, such as SV40 ori, and a multiple cloning site upstream to the sequence of mSEAP. Such plasmid may be used as negative control or to clone a promoter for characterization and thus lacks eukaryotic promoter and enhancer sequences. Alternatively, such reporter plasmid comprises the mSEAP sequence according to the present invention, which is operably linked to a promoter and a enhancer sequence, and are used as positive controls or as a reference for comparing activities of promoters and

enhancers. Any combination of vector, administration method, and/or compositions used for administration can also be selected or optimized through this method. In a preferred embodiment, the invention includes a method of expressing a transgene comprising selecting a vector for expressing the transgene, inserting a nucleic acid comprising, consisting essentially of, or consisting of a mSEAP sequence, such as those of SEQ ID NO: 1 or 2, into the vector, whereby the vector causes the expression of or is capable of expressing a mSEAP polypeptide, administering the vector containing the mSEAP sequence to a cell or mammal, detecting the expression of mSEAP polypeptide for a period of about 40 days or more after administering the vector, employing the selected vector without substantial changes in its nucleotide sequence to deliver a transgene product to a cell or animal. The vector without substantial changes in its nucleotide sequence will be the same vector with optional sequence modifications due to the insertion of a different transgene sequence or use of different insertion sites, or sequence modifications that do not result in a substantial change in the expression of the transgene, or sequence modifications that do not substantially effect the function of the vector. As described for this aspect and any aspect of the invention, the transgene can be any number of sequences that encode a polypeptide or protein or any number of sequences that encode a functional transcript. Functional transcripts include, for example, anti-sense nucleic acids, ribozymes, and other nucleic acids intended to act within a cell. The protein or polypeptide-encoding sequences can be those that encode a therapeutic activity or an activity that performs a function in the cell. Numerous transgenes encompassing these activities have been used and can be selected for use with the invention, and the selection of the transgene itself does not limit the scope or practice of the invention.

25 The vectors that can be used include any cell or composition that allows a nucleic acid to be introduced into a cell. For example, the vectors can be nucleic acids, plasmids, cosmids, recombinant viral vectors, liposomes bearing nucleic acids or recombinant viruses, recombinant or genetically modified cells, and compositions

comprising these examples or any combination of these examples. Furthermore, the nucleic acids and vectors can be used with any cell type, cell line, primary cell culture, tumor cell, or other cell from or derived from an animal. A particular embodiment of a cell comprising an mSEAP-encoding nucleic acid, mSEAP-
5 containing vector, or an mSEAP polypeptide of the invention, is a model cell for studying a disease or the treatment of disease. For example, a model tumor cell expressing an mSEAP of the invention can be inserted into an animal. The model tumor cell can be a human cell, a mouse cell, a rat cell, or any appropriate mammalian or animal cell. A change in mSEAP expression level can be assayed
10 during a variety of treatment regimens or compounds given to the animal. Reduced expression levels, or changes in expression levels, can then detect a treatment or compound that influences tumor growth, volume, or cell metabolism (*see, for example, Nilsson, et al. Cancer Chemotherapy and Pharmacology 49:93-100 (2001)*). Thus, a cell of the invention can be a tumor cell or any other cell inserted into an
15 animal as well as a cultured cell. Both *in vivo* and *in vitro* methods to screen or identify a compound for an effect on cell viability, cell protein expression, cell division, apoptosis, or other property can incorporate or use an mSEAP polypeptide or vector comprising an mSEAP reporter gene of the invention. The present invention is thus directed to a method of monitoring tumor growth and/or response to
20 anticancerous drug treatment, such as anti-angiogenic therapeutics, using the mSEAP of the present invention as the *in vivo* reporter gene in a mouse model in which the tumor cell line is stably transfected with a constitutively expressed mSEAP sequence according to the present invention. The present invention is further directed to a method of monitoring tumor growth comprising injecting athymic nude mice or any
25 susceptible animals with cells from cancer tissue or cell lines, which are stably transfected with the mSEAP of the present invention, and measuring the level of mSEAP to monitor the amount of tumor cells, and tumor growth. In effect, the level of mSEAP is known to be proportional to the number of the tumor cells in the animal or in the culture medium. The present invention is further directed to a method of

monitoring or screening the efficiency of chemotherapeutics and anti-angiogenic therapeutics comprising injecting athymic nude mice or any susceptible animals with cells from cancer tissue or cell lines which are stably transfected with the mSEAP sequence of the present invention, treating said mice or animals with the anticancer drug to be tested, and measuring the level of mSEAP to monitor tumor growth, and thus the efficiency of the anticancer drug treatment. The response to the anticancer treatment may be evaluated by measuring mSEAP levels in the blood during the course of the treatment. For example, the method according to the present invention is advantageous to screen for new anticancer therapeutics against intraperitoneally tumors, such as ovarian carcinomas, or subcutaneous tumors. Chemotherapeutic agents which may be screened for example by using the method according to the present invention include the platinum containing compounds, such as cisplatin, carboplatin, and oxaliplatin, alone or in combination with cyclophosphamide or a taxol analogue paclitaxel, or taxotere. The correlation between the tumor growth and mSEAP levels may be evaluated by measuring the tumor volume after death and dissection of the tumor, or when the tumor is accessible (*i.e.*, subcutaneous) on the animal alive, and measuring the levels of mSEAP in the plasma. Therefore, the levels of mSEAP can provide an appropriate marker of tumor growth and anticancer drug treatment.

The invention also provides nucleic acids that encode AP activity and comprise one or more nucleotide substitution, deletion, or addition changes from a nucleotide sequence encoding a polypeptide having the amino acid sequence of SEQ ID NO: 1 or 2, as well as the AP proteins themselves. The AP activity of these proteins allows for the long term expression analysis noted above, for over about one month, or over about 40 days, or about 60 days, or about 90 days, or about 180 days, or about 270 days. Alternatively, the AP activity in cells or animals with introduced nucleic acid sequences is relatively stable over these periods of time, i.e. the levels do not change more than about 5 fold, or more than about 10 fold from the levels detectable after day 10. These nucleic acids and proteins can be produced by any of numerous

mutation generating techniques known in the art. The proteins and nucleic acids can be tested for their ability to possess or encode the long term expression activity by the same methods or types of methods described throughout this disclosure.

5 **Brief Description of the Drawings**

Figure 1A and 1B depicts a sequence alignment between hSEAP (translated from GI 2190731 of GenBank), the murine full-length alkaline phosphatase (AP) protein, mEAP (translated from GI 192976 of GenBank), and an engineered mammalian AP
10 of the invention, mSEAP. The "Consensus" sequence listed at the bottom line is the same sequence as the mSEAP.

Figure 2A is a plasmid map of pMW18, described in Example 1. It includes the C-terminal truncated form of the EAP coding region.

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Figure 2B is a plasmid map of pMW19, described in Example 1. It includes the C-terminal truncated form of the EAP coding region.

Figure 2C is a plasmid map of pXL3872, described in Example 1. It includes the mSEAP cDNA. Exon 11 has been truncated as described in the examples (listed
20 here as "exon 11 truncated").

Figure 3 shows the expression levels of alkaline phosphatase activity (AP) following introduction of transgene in Balb/C mice. AP activity is measured by sampling 12.5
25 µl of serum with the phosphalight kit (Tropix; Bedford, MA). The boxes represent the expression following introduction of 25µg of plasmid DNA comprising an engineered mSEAP reporter gene of the invention. The triangles represent the expression levels following the introduction of 25µg of the hSEAP reporter gene

known in the art. The circles represent control where no plasmid DNA was used in the same injection vehicle. The vector is introduced to the mice by intramuscular injection enhanced by electric pulses.

- 5 Figure 4 shows the expression levels of alkaline phosphatase activity following introduction of transgene in C57/BL6 mice. The experimental details are the same as in Figure 3. The boxes represent the expression following introduction of 25µg of plasmid DNA comprising the hSEAP reporter gene known in the art. The triangles represent the expression levels following the introduction of 25µg of a mSEAP
10 reporter of the invention. The circles represent control where no plasmid DNA was used in the same vehicle.

- Figure 5 demonstrates the comparable half-life of mSEAP and hSEAP in the bloodstream of Balb/C mice. Two plasmids were co-injected i.m., as per the method
15 of Mir *et al.*, PNAS 96:4262-7 (1999): 25µg of a first plasmid encoding the transcription factor tTA, which is active in the absence of doxycycline; and 25µg of a second plasmid encoding either the mSEAP (pMW19) or encoding the hSEAP. Both of the mSEAP and hSEAP sequences used are under the control of a tTA-responsive promoter. Alkaline phosphatase activity was assessed 7 days after gene transfer,
20 after which mice were given doxycycline by gavage. Thereafter, the mice were given 0.2 mg/ml doxycycline in the drinking water. The triangles represent the percent of AP activity or protein present in the sample of blood following introduction of a mSEAP. The boxes represent the percent of AP activity or protein present in a sample following introduction of hSEAP. The half-life of AP activity or
25 protein falls at nearly the same rate.

Figure 6 shows the results of a method of analyzing long term expression in a mammal. Balb/C mice are injected i.m. with 25µg plasmid DNA encoding mSEAP

under the control of a CMV transcription promoter; or with 25µg plasmid DNA encoding mSEAP under the control of rtTA2M2 in combination with 25µg plasmid DNA encoding rtTA2M2; or with saline as control. See Urlinger *et al.*, *PNAS* 97: 7963-68 (2000). Gene transfer was enhanced by electric pulses according to Mir *et al.* (1999), noted above. Mice were given 0.2 mg/ml doxycycline in the drinking water. The AP activity is assessed at the indicated time points. The boxes represent expression levels following introduction of an mSEAP under the control of the CMV promoter. The triangles represent the expression levels following introduction of an mSEAP under the control of the tTA-responsive promoter.

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Figure 7 shows the amino acid sequence of mSEAP, with underlined amino acids that can be replaced by conservative amino acid substitutions to create exemplary mutants or derivative polypeptides of a mSEAP sequence of the invention or of the sequences of SEQ ID NO: 1 or 2.

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Each of the references (publication, article, web page, information source, GenBank or SwissProt sequence, or patent document, for example) referred to in this specification is hereby specifically incorporated herein by reference, in its entirety. Furthermore, each reference or any combination of references can be relied on and used, in whole or in part, to make, use, and test embodiments of the invention or specific examples described here. As this statement applies to each and every reference, document, or source of information, this specification will not repeat the incorporation by reference. This statement operates to effectively incorporate by reference in their entirety each and every reference (as defined above) listed or referred to in the specification.

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In making and using aspects and embodiments of this invention, one skilled in the art may employ conventional molecular biology, virology, microbiology, and

recombinant DNA techniques. Exemplary techniques are explained fully in the literature and are well known in the art. For example, one may rely on the following general texts to make and use the invention: Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, Second Edition (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York and Sambrook *et al.* Third Edition (2001); *DNA Cloning: A Practical Approach*, Volumes I and II (D.N. Glover ed. 1985); *Oligonucleotide Synthesis* (M.J. Gaited. 1984); *Nucleic Acid Hybridization* (B.D. Hames & S.J. Higgins eds. (1985)); *Transcription And Translation* Hames & Higgins, eds. (1984); *Animal Cell Culture* (R.I. Freshney, ed. (1986)); *Immobilized Cells And Enzymes* (IRL Press, (1986)); B. Perbal, *A Practical Guide To Molecular Cloning* (1984); F.M. Ausubel *et al.* (eds.), *Current Protocols in Molecular Biology*, John Wiley & Sons, Inc. (2001), Coligan *et al.* (eds.), *Current Protocols in Immunology*, John Wiley & Sons, Inc. (2001); Dracopdi *et al.*, *Current Protocols in Human Genetics*, John Wiley & Sons, Inc. (2001), W. Paul *et al.* (eds.) *Fundamental Immunology*, Raven Press; E.J. Murray *et al.* (ed.) *Methods in Molecular Biology: Gene Transfer and Expression Protocols*, The Humana Press Inc. (1991); and J.E. Celis *et al.*, *Cell Biology: A Laboratory Handbook*, Academic Press (1994).

As used herein, a "vector" means any nucleic acid or nucleic acid-bearing particle or composition, cell, or organism capable of being used to transfer a nucleic acid into a host cell. The term "vector" includes both viral and nonviral products and means for introducing the nucleic acid into a cell. A "vector" can be used *in vitro*, *ex vivo*, or *in vivo*. Non-viral vectors include plasmids, cosmids, and can comprise liposomes, electrically charged lipids (cytofectins), DNA-protein complexes, and biopolymers, for example. Viral vectors include retroviruses, lentiviruses, adeno-associated virus, pox viruses, baculovirus, reoviruses, vaccinia viruses, herpes simplex viruses, Epstein-Barr viruses, and adenovirus vectors, for example.

A "nucleic acid" is a polymeric compound comprised of covalently linked nucleotides, from whatever source. Nucleic acid includes polyribonucleic acid (RNA) and polydeoxyribonucleic acid (DNA), both of which may be single-stranded or double-stranded. DNA includes cDNA, genomic DNA, synthetic DNA, and semi-synthetic DNA. The term "nucleic acid" also captures sequences that include any nucleotide base analogue or synthetic nucleotide base.

Percent "identity" between two nucleic acids or two polypeptide molecules refers to the percent defined by a comparison using a basic blast (blastn, blastp, blastx, tblastn, or tblastx, for example) search at the default setting (*see, for example*, NCBI BLAST home page: <http://www.ncbi.nlm.nih.gov/BLAST/>). "Homology" can be determined by a direct comparison of the sequence information between two polypeptide molecules by aligning the sequence information and using readily available computer programs. Alternatively, homology can be determined by hybridization of polynucleotides under conditions allowing for the formation of stable duplexes between homologous regions and detecting the identifying double-stranded nucleic acid.

One or more amino acid residues within a sequence can be substituted by another amino acid of a similar polarity, which acts as a functional equivalent when the substitution results in no significant change in activity in at least one selected biological activity or function. A derivative polypeptide will be a functional equivalent of a given amino acid sequence. For example, one or more substitutions at the positions indicated (by underlining) in Figure 7 can be made in SEQ ID NO: 1 or 2 to produce a functionally equivalent, derivative polypeptide. Truncations from the C-terminal end of SEQ ID NO: 1 or 2 can also be made to produce a functionally equivalent, derivative polypeptide. Conservative substitutions for an amino acid within a sequence may be selected from other members of the class to which the amino acid belongs. For example, the nonpolar (hydrophobic) amino acids include

alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan and methionine. Amino acids containing aromatic ring structures are phenylalanine, tryptophan, and tyrosine. The polar, neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine. The positively charged (basic) amino acids include arginine, lysine and histidine. The negatively charged (acidic) amino acids include aspartic acid and glutamic acid. Alterations of an amino acid with another amino acid from the same class will not substantially effect activity, apparent molecular weight as determined by polyacrylamide gel electrophoresis, or significantly affect the isoelectric point.

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"Isolated," when referring to a nucleic acid, gene, or vector, means that the indicated molecule or product is present in the substantial absence of other biological macromolecules of the same type, or that the indicated molecule or product is present after being subjected to at least one purification step or procedure, such as cell lysis, precipitation, size separation, extraction, chromatography, or any other technique known in the art. Thus, an "isolated nucleic acid molecule that encodes a particular polypeptide" can be to a nucleic acid molecule substantially free of other nucleic acid molecules that do not encode the particular polypeptide. However, the preparation or sample containing the molecule may include other components of different types. In addition, "isolated" can also mean that a particular molecule or product has been purified by at least one purification step or procedure from its ultimate or original source.

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As used herein, a "reporter gene" can refer to a nucleic acid that encodes a detectable polypeptide or protein, such as AP, or can refer to the protein product of that expressed nucleic acid. Thus, the mSEAP reporter gene and protein can mean the genomic DNA encoding EAP modified to become or encode a mSEAP polypeptide, or a modified cDNA encoding a mSEAP, or the mSEAP protein itself (the mSEAP reporter gene product). The novel mSEAP reporter gene and protein of the invention

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specifically include SEQ ID NO: 1, SEQ ID NO: 1 in combination with a mammalian signal sequence at the N-terminal end, SEQ ID NO: 1 in combination with a murine signal sequence at the N-terminal end, SEQ ID NO: 2, any of the above with about 1 to about 5 amino acids deleted from the C-terminal end, and any
5 derivative or mutant of SEQ ID NO: 1 or 2 that has not previously been disclosed to the public or in an application for patent, as well as the nucleic acids that encode these sequences. As used herein, a derivative or mutant can be a sequence with about 80% identity, about 90% identity, or about 95% identity with SEQ ID NO: 1 or
10 2 using a blast comparison at the default settings, as well as nucleotide sequences encoding them. A derivative polypeptide can also possess the long term expression characteristics of being detectable over at least about 40 days, or at least about 60 days, or at least about 180 days, or at least about 270 days after insertion into a cell. The mSEAP polypeptides or reporter gene products of the invention do not include a polypeptide with the exact amino acid sequence of SEQ ID NO: 13.

15 Numerous gene transfer methods and techniques can be used in conjunction with the invention beyond those specifically described. Many of the references listed can be used in selecting an appropriate gene transfer technique, composition, or delivery method. Reporter genes have been used in a number of mammalian test subjects,
20 including mouse, rabbit, cat, dog, primates, and humans. One of skill in the art is familiar with the techniques and methods appropriate for these mammalian test subjects. *See, for example*, Rosenberg *et al.*, *New Eng. J. Med.* 323: 570-78 (1990); Cavazzana-Calvo *et al.*, *Science* 288: 669-72 (2000); Dobson *et al.*, *Brit. Med. J.* 320: 1225 (2000); Buckley *et al.*, *Nature Med.* 6: 623-24 (2000); and the general
25 texts and references listed above.

We have engineered the mouse embryonic alkaline phosphatase (mEAP) gene to be amenable for long term expression studies and the analysis of gene transfer methods and vectors. In so doing, we have addressed or solved several problems relating to

the use of reporter genes in long term expression analysis. For example, the background AP activity in some animal tissue is quite high, for example plasma from mice. Heating plasma samples at 65°C for 5 to 30 minutes can reduce the background from tissue non-specific alkaline phosphatase (TNAP) activity.

- 5 Furthermore, inhibitors, such as L-homoarginine, can be used to reduce background TNAP activity. Pretreatment with heat and/or inhibitors is commonly used with AP activity assays. (Cullen and Malim, Meth. Enzymol. 216:362-368 (1992)). Until now, it was not known whether an engineered form of mEAP would be thermostable enough and resistant enough to TNAP inhibitors for use as a reporter gene.
- 10 Furthermore, it was not known if a reporter gene, and particularly an AP reporter gene, could be expressed and detected over long periods of time. Since the mEAP protein is naturally expressed in embryonic cells in mice, the expression of the engineered mSEAP protein may be subject to an endogenous or physiological regulation that shuts it off, rendering it useless as a reporter gene.

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- The mSEAP reporter gene sequences, nucleic acids, and proteins of the invention are the first that can be expressed and detected over several months in the classical laboratory, immuno-competent mammal after somatic gene transfer. The same vectors and nucleic acids can be used in a number of mammals, including, but not
- 20 limited to, mouse, rat, pig, rabbit, goat, cow, sheep, macaque, cynomolgus macaque, and human. The freedom to use immuno-competent animals or to avoid treatments that inhibit the immune response reduces the complexity of the protocol, reduces the added risk to the animal, and reduces the cost of gene transfer experiments. Thus, the invention provides at least an economic advantage compared to other available
- 25 methods for analyzing long term expression. The invention also provides the advantage of analyzing expression in a physiologically normal patient or animal. In addition, AP activity or the presence of the mSEAP protein can be assayed by several simple techniques, including those employing chemiluminescent, fluorescent, or other detectable substrates and assays.

We have demonstrated the use and advantages of the invention in an intramuscular gene transfer experiment using immuno-competent mice. For example, Figure 3 shows that the level of expression of mSEAP reporter genes of the invention are
5 stable over more than 250 days. In contrast, other reporter genes show a level of expression that drops after 21 days and the levels vary exponentially within the period after the initial ability to detect the activity. The result is consistent in two strains of mice with differing genetic backgrounds (Balb/C and C57/BL6). The lower activity of mSEAP is due to the presence of alkaline phosphatase inhibitors
10 present in the detection kit (PhosphaLight, Tropix; Bedford, MA), which affect mSEAP activity but not hSEAP activity (data not shown).

We have also demonstrated that an immune response against hSEAP is the probable cause of the drop in hSEAP activity because we detected anti-hSEAP antibodies in
15 mice injected with plasmid encoding hSEAP (*see* Table 1).

Table 1

	1	2	3	4	5	6	7	8
	64n g/ml mAb (Si gm a)	Ne g con trol: ser um	C M V- hS E A P #1	C M V- hS E A P #2	C M V- hS E A P #3	C M V- hS E A P #4	CM V- hS EA P #5	pos itiv e con trol: mAb in neg ser um
undil uted	0.4 86	0.0 36	0. 47 3	0. 52	0. 48 8	0. 4	0.5 18	0.5 46
1:2	0.4 87	0.0 36	0. 53 9	0. 52 5	0. 53 2	0. 35 8	0.5 76	0.4 39
1:4	0.4 24	0.0 41	0. 55 1	0. 49 7	0. 53	0. 34 4	0.5 12	0.3 41
1:8	0.2 82	0.0 33	0. 46 9	0. 43 4	0. 48 4	0. 26 4	0.3 95	0.2 09
1:16	0.1 69	0.0 22	0. 32 8	0. 36 9	0. 4	0. 19 9	0.3 71	0.1 23
1:32	0.0 91	0.0 18	0. 27 5	0. 3	0. 30 9	0. 11 8	0.2 62	0.0 71
1:64	0.0 47	0.0 1	0. 16 7	0. 21 7	0. 21 5	0. 07 7	0.1 67	0.0 42
0	0.0 07	0.0 05	0. 00 6	0. 00 6	0. 00 5	0. 00 7	0.0 07	0.0 09

In Table 1, the detection by ELISA of anti-hSEAP antibodies in mice serum after
5 i.m. electrotransfer of 25 µg plasmid containing DNA encoding hSEAP driven by the

cytomegalovirus immediate early gene promoter (CMV) is shown. Microplates are coated with purified hPLAP (SIGMA, St. Louis, MO) and incubated with serum of mice injected with saline (neg serum: row #2) or hSEAP encoding plasmid (CMV-hSEAP, rows #3-7), or with anti-hPLAP monoclonal antibody diluted in PBS (row #1) or serum of non injected mice (row #8). Anti-hSEAP antibodies are detected by an anti-mouse antibody-HRP conjugate. After reaction with TMB the absorbance is read at 450 nm. Comparing the results of the negative control serum, row #2, with those of row # 3-7 shows that for each of the samples where hSEAP plasmid is injected and then expressed (row # 3-7) there are detectable levels of anti-hSEAP antibodies. This indicates that using the hSEAP reporter gene deleteriously results in the production of antibodies, confounding the results of expression assays and inappropriately inducing an immune response in the animal tested.

Furthermore, we have also demonstrated that the stable levels of mSEAP activity shown in Figure 3 is not due to its stability in the bloodstream but rather to its sustained and persistent synthesis or expression by the cells bearing the transgene. In Figure 5, we measure the decay of mSEAP and hSEAP activity after shutting down transcription. To accomplish this, we co-injected mice with two plasmids: one plasmid encoding the transcription factor tTA, which is active in the absence of doxycycline; and the second plasmid encoding mSEAP (pMW19) or hSEAP under the control of a tTA-responsive promoter (*see, for example, Urlinger, et al., PNAS 97: 7963-68 (2000)*). The mice are given doxycycline 7 days after gene transfer and the alkaline phosphatase activity assessed thereafter.

The results in Figure 5 show that the half-life of mSEAP activity is less than 2 days and that the half-life of hSEAP activity is less than 4 days. These numbers take into account the half-life of the reporter protein, of its mRNA, and the decay of the transcription rate upon doxycycline administration. Hence, the sustained mSEAP

activity shown in Figure 3 cannot be explained by a prolonged half-life of mSEAP in the bloodstream.

The utility of this invention is further demonstrated by the experiment presented in Figure 6. We assessed the impact of the chimeric transcription factor rtTA2M2 (*see, for example, Urlinger, et al., PNAS 97: 7963-68 (2000)*) on the duration of mSEAP expression. In this experiment, mice are injected with a plasmid expressing mSEAP under the control of a constitutive transcription promoter (CMV) or with a plasmid expressing mSEAP under the control of an rtTA2M2-responsive promoter in combination with an rtTA2M2-encoding plasmid. Animals are given doxycycline (a water-soluble derivative of tetracycline) in the drinking water because rtTA2M2 is activated by this molecule. This experiment demonstrates clearly that rtTA2M2 is not capable of sustaining high levels of expression of mSEAP for more than 15 days. This may be due to a cytotoxic immune response against the cells expressing rtTA2M2 and mSEAP (rtTA2M2 is a fusion protein made of domains of bacterial and viral origins). In contrast, the CMV-driven expression of mSEAP results in stable levels of expression. Thus, the method of expressing a transgene or the method of analyzing long term expression of a transgene provided by this invention allows one to select appropriate regulatory elements and vectors for long term expression applications. Numerous regulatory elements exist in the art and can be selected for use and analysis. The invention is not limited to the use of any particular regulatory element or those specifically exemplified here.

The results discussed above and in the following specific examples are merely representative of the scope of the invention and content of this disclosure. One skilled in the art can use the information here to devise, produce, and use additional embodiments of the invention. Thus, the examples given here should not be taken as a limitation on the scope or extent of the invention.

Example 1. Production of Plasmids Comprising AP Activity-encoding Nucleic Acids

A nucleic acid encoding the mSEAP reporter gene or polypeptide of the invention
 5 can be derived from mouse genomic DNA, synthesized from a GenBank sequence,
 or derived from one of the plasmids containing an *EAP* gene (for example, pSVT7-
EAP from Narisawa *et al.*, Development 116:159-165 (1992), Manes *et al.*,
 Genomics 8: 541-554 (1990); or those of Hahnel *et al.*, Development 110: 555-564
 (1990); Bao *et al.*, Gynecologic Oncology 78:373-379 (2000); Berger *et al.*, Gene
 10 66:1-10 (1988)), or derived from a mammalian AP gene or cDNA or other nucleic
 acid (for example, mouse secreted embryonic phosphatase – such as ACCESSION
 AY054302; mouse alkaline phosphatase 5 – such as ACCESSION NM_00743;
 human intestinal alkaline phosphatase – such as ACCESSION M31008 M15184;
 human adult intestinal alkaline phosphatase – such as ACCESSION M15694; human
 15 intestinal alkaline phosphatase – such as ACCESSION NM_00631; rat membrane
 associate intestinal alkaline phosphatase – such as ACCESSION X17611 S51096;
 human alkaline phosphatase – such as ACCESSION X55958; human placental
 alkaline phosphatase – such as ACCESSION M13077; rat intestinal alkaline
 phosphatase 1 – such as ACCESSION NM_022665; human placental like alkaline
 20 phosphatase – such as ACCESSION X53279; human placental alkaline phosphatase
 type 1 – such as ACCESSION M14169; pSEAP-Enhancer – ACCESSION U09662;
 pSEAP-Promoter – ACCESSION U09663; pSEAP2-Promoter – ACCESSION
 U89940; human clone MGC:5096 IMAGE:3460735 – such as ACCESSION
 BC009647; pSEAP-Basic – such as ACCESSION U09660; bovine intestinal
 25 alkaline phosphatase III – such as ACCESSION AF052226; canine intestinal
 alkaline phosphatase – such as ACCESSION AF250845; feline alkaline phosphatase
 – such as ACCESSION U31569). From either the cloned gene sequence, the
 genomic sequence, the cDNA, or other nucleic acid, appropriate PCR oligos are

prepared that result in the deletion of about 22 codons from those immediately preceding the stop codon. The determination of the number of codons to delete or truncate from the C-terminus depends on the functional properties of the resulting polypeptide. Functionally, the deletion of the C-terminal codons removes the membrane anchoring activity possessed by the native C-terminal amino acids. Accordingly, any C-terminal truncation resulting in a polypeptide that functionally lacks a membrane anchoring activity can be used and is included in this invention.

In one way of truncating the C-terminus, a stop codon is added or supplied at the desired end of the coding region. For example, a mouse genomic fragment encoding EAP can be amplified by PCR with oligonucleotides A and B below to generate the mSEAP of SEQ ID NO: 2. Alternatively, oligos A and C can be used to generate the full-length EAP encoding fragment. The full-length fragment can be used to create mutations or as a control.

A: 5'-GTATAAGCTTGCCACCATGTGGGGAGCCTGCTTGCTGCTGC-3'
(SEQ ID NO: 4)

B: 5'-GTTTTCTAGATCAGCCCGGGCTCACTGCACTGCTCTGG-3'
(SEQ ID NO: 5)

C: 5'-ACTCTCTAGATCAGGGTTCAGCCGCCGCCATCAGC-3'
(SEQ ID NO: 6)

In one method, the PCR fragments are cut by *Hind*III and *Xba*I and inserted between the *Hind*III and *Avr*II sites of pXL3443 (a pBKSII-derived plasmid containing the CMV I/E promoter and the SV40 late poly(A) signal separated by a multiple cloning site) to generate pMW12 and pMW18 (see Figure 2A). The same insert can be used

between the *Hind*III and *Xba*I sites of pTRE2 (Clontech, Palo Alto, CA) to generate pMW19 (see Figure 2B). Plasmid pMW12 harbors the native *EAP* gene and pMW18 the genomic clone with a truncation at the C-terminal end of the *EAP* coding region, both under the control of the CMV I/E promoter (from about -522 to about +74 from the start), and the SV40 late poly(A) signal. Plasmid pMW19 directs the transcription of the truncated murine *EAP* gene from a CMV I/E minimal promoter (from about -51 to about +70 from the start) under the control of a tetracycline response element (see, for example, Gossen and Bujard, PNAS 89: 5547-5551 (1992); (Urlinger, *et al.*, PNAS 97: 7963-68 (2000)), with a β -globin poly(A) signal.

A cDNA encoding mSEAP of SEQ ID NO: 2 can be obtained as follows. Murine C2C12 myoblasts are transiently transfected with pMW18 complexed to LipofectAMINE (Invitrogen - Life Technologies, Gaithersburg, MD) according to the manufacturer's protocol. PolyA⁺ RNA is extracted from transfected cells with a commercial kit (Dynal dynabeads mRNA direct kit). PolyA⁺ RNA is reverse-transcribed and amplified by PCR (RT-PCR kit; Promega, Madison, WI) with oligonucleotides C9415 and C9416, shown below.

C9415: 5'-CGCGAAGCTTGCCACCATGTGGGGAGCCTGCTTGC-3'
(SEQ ID NO: 7)

C9416: 5'-CTCTTCTAGACTATCAGCCCGGGCTCACTGCACTGC-3'
(SEQ ID NO: 8)

Oligos C9415 and C9416 are designed to amplify the mSEAP nucleic acid flanked at the 5' end by a *Hind*III site, and flanked at the 3' end by an *Xba*I site. The product of the RT-PCR reaction is ligated in pGEMT-easy (Promega, Madison, WI) and

- transformed into *E. coli* DH5 α . The resulting plasmid contains the mSEAP cDNA surrounded by a multiple cloning site. A plasmid pCOR (see Soubrier *et al.*, Gene Therapy 6: 1482-88 (1999)) can then be used to construct a plasmid pXL3872 (Figure 2C) containing the mSEAP nucleic acid under the control of the CMV I/E promoter (-522/+74) and the SV40 late poly(A) signal using the *Hind*III to *Xba*I sites of pXL3856 (a pCOR plasmid containing the CMV I/E promoter (-522/+74) and the SV40 late poly(A) signal). The expression cassette region of plasmid pXL3872 (Figure 2C) is verified by sequencing.
- 10 Plasmids encoding other AP sequences are constructed in like manner for control AP proteins, for other mSEAP polypeptides of the invention, or derivative mSEAP polypeptides of the invention. For example, pXL3402 is a pCOR (Soubrier *et al.*, 1999) plasmid with the SEAP cDNA under the control of the CMV I/E promoter (-522/+74) and the SV40 late poly(A) signal. Other plasmids put the SEAP under the control of the tetracycline responsive elements (TRE) and the SV40 late poly(A) signal. In another example, the *Pst*I-*Spe*I fragment encoding luciferase in pBi-L (Baron *et al.*, Nucleic Acids Res. 23:3605-06 (1995)) can be replaced by nucleic acid encoding an AP activity flanked by the *Pst*I and *Spe*I sites. A PCR amplified fragment from pSEAP2-basic (Clontech) can be used. Also, a plasmid with an AP activity under the control of the CMV I/E promoter and the SV40 late poly(A) signal can be constructed by replacing the *Sac*I-*Pvu*II fragment of pBi-L (TRE) by the *Sac*I-*Stu*I fragment of pXL3031 (CMV I/E promoter, Soubrier *et al.*, 1999) and subsequently replacing the *luciferase* ORF by the *SEAP* ORF.
- 20
- 25 The plasmids can be prepared for gene transfer administration by purification with the endo-free Mega-Prep kit (Qiagen, Germantown. MD). Preferably, the endotoxin level detected in the samples is less than 20 EU per mg of DNA. Other methods and

techniques for purifying vectors and nucleic acids for administration to a mammal are known in the art and can be used.

5 Example 2: Transfection of Cultured Cells

C2C12 (ATCC: CRL1772), and HEK293 (ATCC: CRL1573) cells are seeded in 24-well plates (7.5×10^4 cells per well) and grown for 24 h in DMEM supplemented with 10% FCS. Cells are then washed in DMEM without serum and transfected in
10 triplicate by adding to the cells 0.5 ml of OptiMEM mixed with various quantities of AP-encoding plasmid, supplemented to 500 ng with a carrier plasmid and LipofectAMINE (2 μ l for C2C12, 3 μ l for HEK293). Five hours later, the medium containing the DNA and the LipofectAMINE is replaced by 1 ml of DMEM
15 supplemented with FCS (2% for C2C12, 10% for HEK293). Aliquots of the culture medium are collected 2 days post-transfection and frozen at -70°C for storage. The cells are rinsed twice with PBS, incubated with 100 μ l of 0.2% Triton X-100, 50 mM Tris-HCl pH 7.4, 150 mM NaCl, detached from the plate with a scraper and homogenized by repeated pipetting. The lysate is centrifuged 2 minutes at maximum speed in an eppendorf tabletop centrifuge, and the supernatant stored at -70°C .

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Example 3: Intramuscular Gene Transfer

Eight-week-old female Balb/C or C57BL/6 mice (Charles River Laboratories, Wilmington, MA) are anesthetized by intraperitoneal injection of 200 μ l ketamine
25 (8.66 mg/ml) mixed with xylazine (0.31 mg/ml) in 150 mM NaCl. The hind legs are shaved. Twenty-five microliters of a nucleic acid vector containing solution in 150 mM NaCl are injected in the tibialis cranialis muscle. Thirty seconds after injection, transcutaneous electric pulses can be applied through stainless steel parallel

electrodes connected to an Electro Square Porator, Model T820 (BTX, San Diego, CA), and a TDS 210 oscilloscope (Tektronix, Oregon). If used, the plate electrodes are placed on each side of the leg (plate separation distance 4 mm) and 8 square wave pulses (80 volts, 20 ms each pulse, 1 pulse per second) are applied (Mir *et al.*, 1999).

At different time points relative to DNA injection, 50 µl blood samples are collected from the saphenous vein into heparinized capillary tubes (Hem *et al.*, Lab. Anim. 32:364-68 (1998)). Samples are spun 20 minutes at 2000 rpm in a clinical centrifuge and plasma collected and frozen at -70°C for storage.

Example 4: Reporter Gene Expression Analysis

Exemplary Enzymatic Assay

Detection of the AP activity can be carried out with the Phospha Light kit (Tropix, Perkin-Elmer), following the manufacturer's instructions or by using the modifications of Cullen and Malim, Meth. Enzymol. 216: 362-8 (1992)). Fifteen (15) µl of plasma or cell culture medium or cell extract are diluted into 45 µl of 1X dilution buffer (50 mM Tris-HCl pH 7.4; 150 mM NaCl), heated at 65°C for 5 minutes, then cooled down to 4°C in a PCR block thermocycler. Fifty (50) µl of diluted sample are incubated 5 minutes at room temperature with 50 µl of assay buffer (1M diethanolamine pH 10.5-11, 1mM MgCl₂, 10 mM L-homoarginine) before adding 50 µl of reaction buffer (CSPD substrate with Emerald enhancer). The reaction is then incubated 20 minutes at room temperature before chemiluminescence measurement (10 seconds per well) in a MLX microtiter plate luminometer (Dynex).

Exemplary ELISA Assay

Detection of antibodies directed against AP, for example the hSEAP in Table 1, can be performed using an ELISA assay. However, similar assays for other AP proteins, such as those of the invention, can also be used. Wells of a PVC microtiter plate are coated with 50 μ l of a 0.5 μ g/ml solution of human placental alkaline phosphatase (PLAP, Sigma) in 0.2 M $\text{Na}_2\text{CO}_3/\text{NaHCO}_3$ pH 9.5 and incubated overnight at 4°C. The plate is washed 3 times with 250 μ l of 0.1 M phosphate buffer pH 7.5, 0.1 M NaCl (PBS), 0.05% Tween 20 (w/v). The remaining sites for protein binding on the PVC plate are saturated by incubation with PBS, 2% BSA (w/v) for 2 hours at room temperature. Fifty microliters of test plasma or positive or negative controls are added per well and incubated 2 hours at room temperature. The negative control can be the plasma of untreated Balb/c mouse. The positive control is obtained by spiking the plasma of a negative control mouse with 64 ng/ml of a mouse monoclonal antibody to PLAP (clone 8B6, Sigma Immunochemicals). Plasma samples are tested at dilutions 1:1 to 1:64 (two-fold serial dilutions in PBS). Samples are then incubated 1 hour at room temperature with a goat anti-rabbit IgG conjugated to horseradish peroxidase (Bio-Rad) in PBS, 2% BSA (w/v), 0.05% Tween 20, and washed 3 times with 250 μ l of PBS, 0.05% Tween 20. The samples are reacted with 100 μ l 3,3',5,5'-tetramethylbenzidine (USB, Ohio) for 15 minutes at room temperature, and the reaction stopped by adding 50 μ l of 0.5 N H_2SO_4 . The absorbance is read at 450 nm.

Example 5: Long Term Expression Analysis

Mice are injected i.m. with 25 μ g plasmid DNA encoding hSEAP, mSEAP of the invention, or saline control. Gene transfer is enhanced by electric pulses according to Mir, *et al.*, PNAS 96:4262-7 (1999). Blood samples are collected in heparinized capillaries at the indicated time points by saphenous vein puncture, spun 20 minutes

at 2000 rpm in a clinical centrifuge, and 12.5 μ l of serum assessed for alkaline phosphatase activity according to the instructions in the phosphalight kit (Tropix), except that diluted plasma samples are heated 5 minutes at 65°C. Results for exemplary experiments of this type are shown in Figure 3. The mSEAP levels, at the top of the chart, are detected in the first sample after gene transfer and remain consistently high throughout the entire sampling period (over 9 months). In contrast, the hSEAP reporter gene can be detected at levels above control for only about 20 days, and the levels vary greatly. The levels and relative errors reported indicate that the inventive mSEAP reporter genes can be used for extremely long expression studies and that the levels of expression are consistent and reproducible.

Example 6: Preparation of mSEAP-encoding Nucleic Acids and Derivative Polypeptides; Analyzing Expression Vectors

The nucleic acids derived from the mEAP gene noted in Example 1 can be used as the starting point to generate conservative amino acid substitution mutants and derivatives of the mSEAP proteins of the invention. The mSEAP encoding sequence is PCR amplified with an oligo that incorporates one or more nucleotide changes that result in amino acid substitutions. For example, the method described in Ausubel *et al.* Current Protocols in Molecular Biology (chapter 3 and unit 3.17, and chapter 8 and unit 8.5, in particular) can be adapted. The amino acid positions noted by the underlining in Figure 7 represent exemplary sites for conservative amino acid changes or amino acid substitutions and the positions can be correlated to the sequence of any selected alkaline phosphatase activity-encoding nucleic acid used. For example, positions 358, 357, and/or 356 can be modified to encode an isoleucine in place of a valine, and/or a glutamic in place of an aspartic, and/or an arginine in place of a lysine. Once inserted into the sequence, the substitution mutant sequence can be incorporated into a plasmid as in Example 1. The mutant or derivative mSEAP protein can then be expressed as in Example 5 and tested to confirm long

term expression and/or the ability to be used in immuno-competent mammals. Any derivative polypeptide possessing the long term expression characteristics noted above and throughout this disclosure is included in the invention. Testing for these characteristics can be performed as described here. Preferably, the derivative
5 polypeptide will possess about 98%, or about 95%, or about 90%, or about 85%, or about 80%, or about 75% amino acid identity to SEQ ID NO: 1 or 2 in a blast comparison at default parameters.

Exemplary derivative polypeptides can be made using the same techniques used in
10 Example 1 to generate mSEAP nucleic acids. The 3' primer "B", SEQ ID NO: 5, encodes a stop codon resulting in a 22 amino acid truncation in the incorporated PCR amplified product with the A primer, SEQ ID NO: 4.

Translated B primer (SEQ ID NO: 5) 3'5' Frame 2

15 Q S S A V S P G Stop S R K

Selecting a new 3' primer by sliding the stop codon further into the coding sequence results in amplified products that contain fewer codons. Thus, truncated products are produced from the SEAP or EAP cDNAs or genomic clones nucleotides that
20 correspond to 27 amino acid truncations from the murine EAP gene noted in Manes *et al.*, Genomics 8:541-554 (1990). Preferred truncations of about 22 to about 27 amino acids from the mEAP C-terminal end are made in this way. Other truncations are possible, as one of skill in the art appreciates. Furthermore, other deletions and/or substitutions, with or without the C-terminal truncation, can also be selected.
25 The resulting, amplified DNA is then incorporated into a mammalian expression vector, administered to an immuno-competent mouse, and the long term expression characteristics of the AP-activity is tested by the chemiluminescent assay (Tropix; Bedford, MA), for example. Preferred expression vectors include viral vectors, such as adenovirus, adeno-associated virus, or retrovirus, or plasmid vectors. By testing

the AP activity using particular expression vectors or regulatory sequences, an appropriate vector can be selected or optimized for transgene expression. Ligand-dependent regulatory sequences can also be tested for their ability to control expression over long periods of time (*see, for example*, Abruzzese, *et al.*, Hum. Gene Ther. 10:1499-1507 (1999); Urlinger *et al.*, PNAS 97: 7963-68 (2000)), such as the 40 day, 60 day, 90 day, 120 day, 180 day, or 270 day periods possible through the use of the invention.

The invention described and exemplified above is not limited to the specific embodiments and examples presented here. One skilled in the art can use the techniques and knowledge available through the documents and references noted and specifically incorporated herein, or other documents or references, to make and use additional embodiments. Thus, the description above should not be taken as a limitation of the scope or content of this invention.

We claim:

1. An isolated nucleic acid comprising a nucleotide sequence encoding a mammalian alkaline phosphatase activity and having the amino acid sequence of SEQ ID NO: 1 or 2, wherein the nucleic acid does not encode a polypeptide of the identical sequence of amino acids listed in SEQ ID NO: 13.
2. An expression vector comprising the nucleic acid of claim 1.
3. A viral vector comprising the nucleic acid of claim 1.
4. A plasmid vector comprising the nucleic acid of claim 1.
5. A mammalian cell comprising a nucleic acid of claim 1.
6. A mammalian cell comprising a vector of claim 2.
7. A mammalian cell comprising a vector of claim 3.
8. A mammalian cell comprising a vector of claim 4.
9. The cell of claim 5 that is a mouse cell.
10. The cell of claim 5 that is a human cell.
11. The cell of claim 5 that is a primate cell.
12. An isolated nucleic acid having a sequence that encodes a mammalian alkaline phosphatase activity-possessing polypeptide, wherein the polypeptide is capable of being expressed or detected in a mammal for a period of about 40 days, and wherein the nucleic acid encodes a polypeptide comprising the amino acid sequence of SEQ ID NO: 1 or 2 but not that identical to SEQ ID NO: 13, a derivative or mutant of SEQ ID NO: 1 or 2 that is not identical to SEQ ID NO: 13, or the amino acid sequence of SEQ ID NO: 1 or 2 further comprising at least one amino acid substitution that is not identical to SEQ ID NO: 13.
13. The nucleic acid of claim 12, wherein the polypeptide is capable of being expressed or detected in a mammal for a period of about 60 days.
14. The nucleic acid of claim 12, wherein the polypeptide is capable of being expressed or detected in a mammal for a period of about 90 days.
15. The nucleic acid of claim 12, wherein the polypeptide is capable of being expressed or detected in a mammal for a period of about 180 days.

16. The nucleic acid of claim 12, wherein the polypeptide is capable of being expressed or detected in a mammal for a period of about 270 days.
17. An expression vector comprising the nucleic acid of claim 8.
18. A viral vector comprising the nucleic acid of claim 12.
- 5 19. A plasmid vector comprising the nucleic acid of claim 12.
20. A mammalian cell comprising a nucleic acid of claim 12.
21. The cell of claim 20 that is a mouse cell.
22. The cell of claim 20 that is a human cell.
23. The cell of claim 20 that is a primate cell.
- 10 24. A mSEAP reporter gene polypeptide, wherein the polypeptide is capable of being expressed in a mammalian cell and of being detected in a mammal about 40 days after first being expressed.
25. The polypeptide of claim 24, wherein the polypeptide has the amino acid sequence of SEQ ID NO: 1 or 2.
- 15 26. The polypeptide of claim 24, wherein the polypeptide has a sequence with at least 98% amino acid identity to SEQ ID NO: 1 or 2.
27. The polypeptide of claim 24, wherein the polypeptide has a sequence with at least 95% amino acid identity to SEQ ID NO: 1 or 2.
28. The polypeptide of claim 24, wherein the polypeptide has a sequence
20 with at least 90% amino acid identity to SEQ ID NO: 1 or 2.
29. The polypeptide of claim 24, wherein the polypeptide has a sequence with at least 85% amino acid identity to SEQ ID NO: 1 or 2.
30. The polypeptide of claim 24, wherein the polypeptide has a sequence with at least 80% amino acid identity to SEQ ID NO: 1 or 2.
- 25 31. The polypeptide of claim 24, wherein the polypeptide has a sequence with at least 75% amino acid identity to SEQ ID NO: 1 or 2.
32. A cell of a mammal or derived from a mammal comprising the mSEAP polypeptide of claim 24.
33. A mouse comprising a cell of claim 32.

34. A macaque comprising a cell of claim 32.
35. A primate comprising a cell of claim 32.
36. A human comprising a cell of claim 32.
37. A method of expressing a transgene in a mammal comprising
5 selecting a mammalian expression vector, inserting a mSEAP reporter gene nucleic acid into the vector, wherein the nucleotide sequence of the mSEAP nucleic acid encodes a sequence comprising SEQ ID NO: 1 or 2 but not a sequence identical to the sequence of SEQ ID NO: 13, administering the vector to a mammal, whereby the mSEAP reporter gene product is expressed in the mammal, detecting alkaline
10 phosphatase activity or protein from the mSEAP reporter gene at about 30 days or more after administering the vector, inserting a nucleic acid encoding the transgene into the selected mammalian expression vector, or a vector substantially similar to the selected mammalian expression vector, and introducing the resulting vector comprising the nucleic acid encoding the transgene to a cell or mammal.
- 15 38. The method of claim 37, wherein the mammal is a mouse.
39. The method of claim 37, wherein the mouse is a Balb/C or C57/BL6 mouse.
40. The method of claim 37, wherein the mammal is an immuno-competent mammal or a mammal that has not been rendered immuno-deficient.
- 20 41. The method of claim 37, wherein the vector containing the transgene is administered to a human.
42. The method of claim 37, wherein the cell is a human cell.
43. The method of claim 37, wherein the cell is a C2C12 cell, 293 cell, or HEK293 cell.
- 25 44. The method of claim 37, wherein the cell is a primate cell.
45. The method of claim 37, wherein the cell is a mouse cell.
46. The method of claim 37, wherein the detecting of alkaline phosphatase activity or protein from the mSEAP reporter gene is at about 40 days.

47. The method of claim 37, wherein the detecting of alkaline phosphatase activity or protein from the mSEAP reporter gene is at about 60 days.
48. The method of claim 37, wherein the detecting of alkaline phosphatase activity or protein from the mSEAP reporter gene is at about 90 days.
- 5 49. A fusion protein consisting essentially of the amino acid sequence of SEQ ID NO: 1 or 2 wherein the fusion protein does not contain the identical amino acid sequence of SEQ ID NO: 13.
50. A nucleic acid encoding the fusion protein of claim 49.
51. A vector comprising the nucleic acid of claim 50.
- 10 52. A polypeptide comprising the amino acid sequence of SEQ ID NO: 1 linked to a mammalian signal sequence.
53. The polypeptide of claim 52, wherein the signal sequence is selected from SEQ ID NO: 3, 9, 10, 11, or 12.
54. An isolated nucleic acid encoding the polypeptide of claim 52.
- 15 55. An isolated nucleic acid encoding the polypeptide of claim 53.
56. An isolated nucleic acid encoding an amino acid sequence consisting of SEQ ID NO: 1.
57. A nucleic acid encoding an amino acid sequence having 98% identity to the nucleic acid of claim 56.
- 20 58. A nucleic acid encoding an amino acid sequence having 95% identity to the nucleic acid of claim 56.
59. A nucleic acid encoding an amino acid sequence having 90% identity to the nucleic acid of claim 56.
60. A nucleic acid encoding an amino acid sequence having 85% identity to the nucleic acid of claim 56.
- 25 61. An isolated nucleic acid encoding an amino acid sequence consisting of SEQ ID NO: 2.
62. A nucleic acid encoding an amino acid sequence having 98% identity to the nucleic acid of claim 61.

63. A nucleic acid encoding an amino acid sequence having 95% identity to the nucleic acid of claim 61.
64. A nucleic acid encoding an amino acid sequence having 90% identity to the nucleic acid of claim 61.
- 5 65. A nucleic acid encoding an amino acid sequence having 85% identity to the nucleic acid of claim 61.

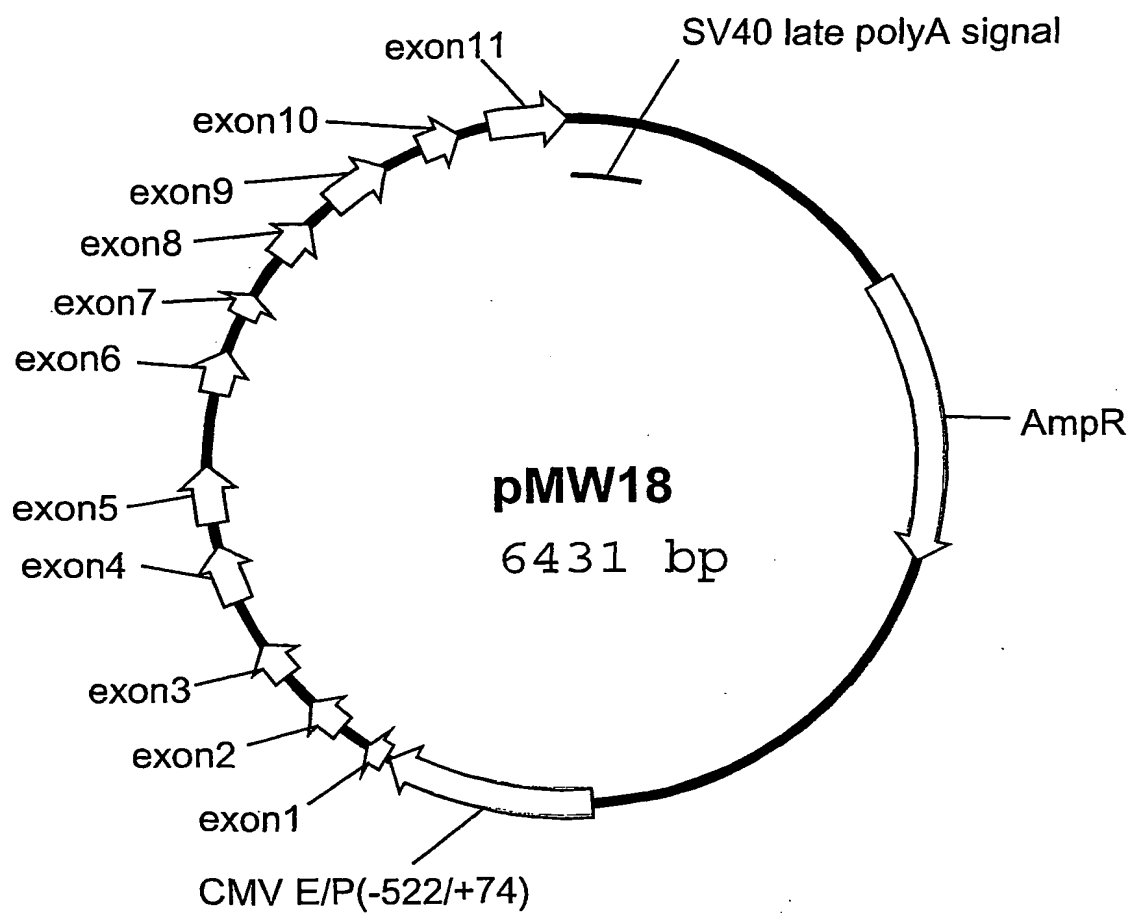
Figure 1A

	(1) 1	10	20	30	40	50	60
hSEAP	(1) -MLLLLLGLRLQLSLG	ILPVEEENPD	FWNREAAE	ALGA	AKKLQ	PAQTAA	AKNLIIIFLGD
mEAP	(1) MWGACLLLLGLSLQV	CPSVIPVEEEN	PAFWNRKAA	EALDA	AKKLK	PIQTS	AKNLVILMGD
mSEAP	(1) MWGACLLLLGLSLQV	CPSVIPVEEEN	PAFWNRKAA	EALDA	AKKLK	PIQTS	AKNLVILMGD
Consensus	(1) MWGACLLLLGLSLQV	CPSVIPVEEEN	PAFWNRKAA	EALDA	AKKLK	PIQTS	AKNLVILMGD
	(61) 61	70	80	90	100	110	120
hSEAP	(60) GMGVSTVTAA	ILKGQKDKLGP	EIPLAMDRFP	YVALSKT	YNVDKH	VPSGATA	TAYLCCG
mEAP	(61) GMGVSTVTATR	ILKGQQQGH	LGPETQLAMDRFP	PHMALSKT	YNTDKQ	I PDS	AGTGTAF
mSEAP	(61) GMGVSTVTATR	ILKGQQQGH	LGPETQLAMDRFP	PHMALSKT	YNTDKQ	I PDS	AGTGTAF
Consensus	(61) GMGVSTVTATR	ILKGQQQGH	LGPETQLAMDRFP	PHMALSKT	YNTDKQ	I PDS	AGTGTAF
	(121) 121	130	140	150	160	170	180
hSEAP	(120) VKGNFQTI	GLSAAARFNQ	CNTTRGNE	VISVMNR	AKKAGK	SVGVVTT	TRVQHASPAGTYAH
mEAP	(121) VKTNMKVI	GLSAAARFNQ	CNTTWGNE	VSVVMHR	AKKAGK	SVGVVTT	TSVQHASPAGTYAH
mSEAP	(121) VKTNMKVI	GLSAAARFNQ	CNTTWGNE	VSVVMHR	AKKAGK	SVGVVTT	TSVQHASPAGTYAH
Consensus	(121) VKTNMKVI	GLSAAARFNQ	CNTTWGNE	VSVVMHR	AKKAGK	SVGVVTT	TSVQHASPAGTYAH
	(181) 181	190	200	210	220	230	240
hSEAP	(180) TVNRNWYS	DADVPASARQ	EGCQDIATQ	LISNMD	IDVILGG	GRKYMFR	MGT PDPEY PDDYS
mEAP	(181) TVNRGWYS	DAQMPASALQ	DGCKDISTQ	LISNMD	IDVILGG	GRKFMFP	KGT PDQEYPTDTK
mSEAP	(181) TVNRGWYS	DAQMPASALQ	DGCKDISTQ	LISNMD	IDVILGG	GRKFMFP	KGT PDQEYPTDTK
Consensus	(181) TVNRGWYS	DAQMPASALQ	DGCKDISTQ	LISNMD	IDVILGG	GRKFMFP	KGT PDQEYPTDTK
	(241) 241	250	260	270	280	290	300
hSEAP	(240) QGGTRLDG	KNLVQEWLAK	RQGARVYV	WNRTELMQ	ASLDPS	VTHLMGL	FEPGDMKYEIHHRDS
mEAP	(241) QAGTRLDG	RNLVQEWLAK	HQGARVYV	WNRSELIQ	ASLNR	SVTHLMGL	FEPNDMKYEIHHRDP
mSEAP	(241) QAGTRLDG	RNLVQEWLAK	HQGARVYV	WNRSELIQ	ASLNR	SVTHLMGL	FEPNDMKYEIHHRDP
Consensus	(241) QAGTRLDG	RNLVQEWLAK	HQGARVYV	WNRSELIQ	ASLNR	SVTHLMGL	FEPNDMKYEIHHRDP

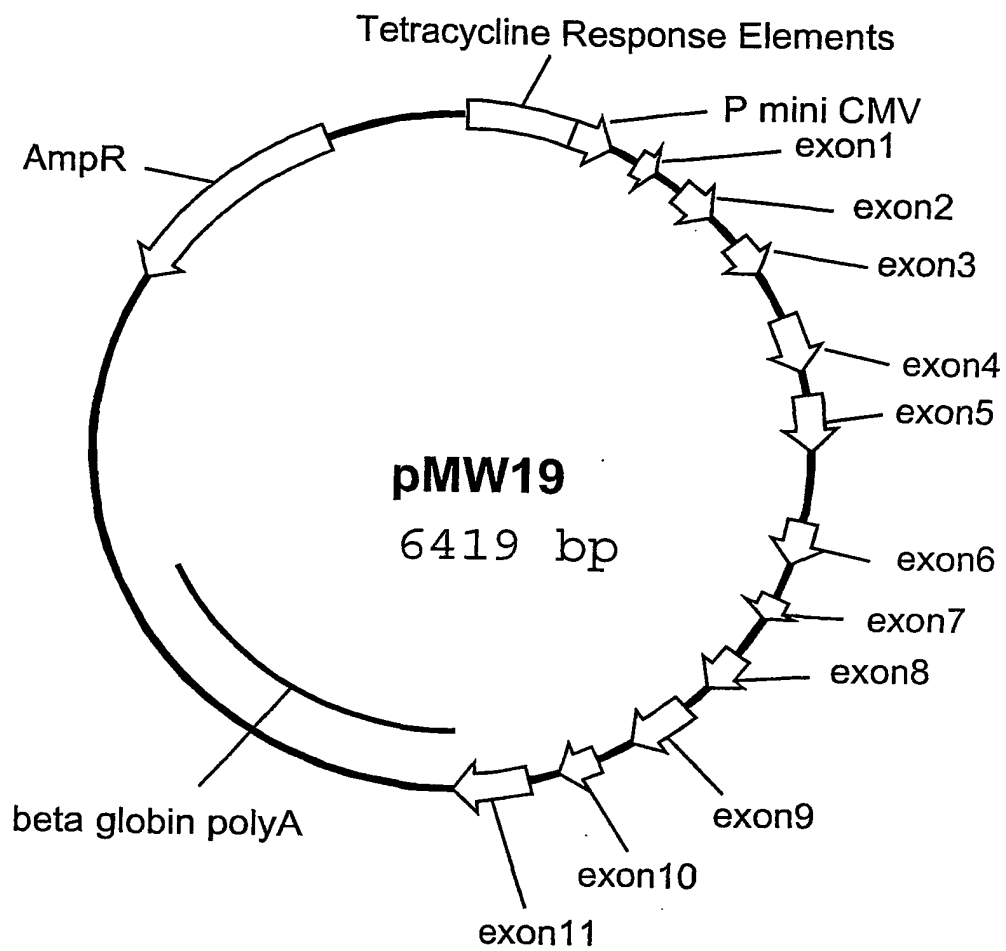
Figure 1B

(301) 301	310	320	330	340	350	360
hSEAP(300)	TLDPSLMEMTEAALRLLSRNPRGFFLFVEGGRIDHGHHSRAYRALTETIMFDDAIERAG					
mEAP(301)	AQDPSLAEMTEVAVRMLSRNPKGFYLFVEGGRIDHGHHETVAYRALTEAVMFDSAVDKAD					
mSEAP(301)	AQDPSLAEMTEVAVRMLSRNPKGFYLFVEGGRIDHGHHETVAYRALTEAVMFDSAVDKAD					
Consensus(301)	AQDPSLAEMTEVAVRMLSRNPKGFYLFVEGGRIDHGHHETVAYRALTEAVMFDSAVDKAD					
(361) 361	370	380	390	400	410	420
hSEAP(360)	QLTSEEDTSLVTADHSHVFSFGGYPLRGSSIFGLAPGKARDRKAYTVLLYGNPGGYVLK					
mEAP(361)	KLTSSEQDTMILVTADHSHVFSFGGYTQRGASIFGLAPFKAEDGKSFTSILYGNPGGYKLH					
mSEAP(361)	KLTSSEQDTMILVTADHSHVFSFGGYTQRGASIFGLAPFKAEDGKSFTSILYGNPGGYKLH					
Consensus(361)	KLTSSEQDTMILVTADHSHVFSFGGYTQRGASIFGLAPFKAEDGKSFTSILYGNPGGYKLH					
(421) 421	430	440	450	460	470	480
hSEAP(420)	DGARPDVTESESGSPEYRQQSAVPLDEETHAGEDVAVFARGPQAHLVHGVQEQTFFIAHVM					
mEAP(421)	NGARADVTEEESSNPTYQQQACVPLSSETHSGEDVAIFARGPQAHLVHGVQEQNYIAHVM					
mSEAP(421)	NGARADVTEEESSNPTYQQQAAVPLSSETHSGEDVAIFARGPQAHLVHGVQEQNYIAHVM					
Consensus(421)	NGARADVTEEESSNPTYQQQAAVPLSSETHSGEDVAIFARGPQAHLVHGVQEQNYIAHVM					
(481)	490	500	510	520	530	
hSEAP(480)	AFAACLEPYTACDLAPPAGTTDAAHPG					
mEAP(481)	AFAACLEPYTDCGLASPAGQSSAVSPGYMSTLLCLLAGKMLMLMAAAEP-					
mSEAP(481)	AFAACLEPYTDCGLASPAGQSSAVSPG					
Consensus(481)	AFAACLEPYTDCGLASPAGQSSAVSPG					

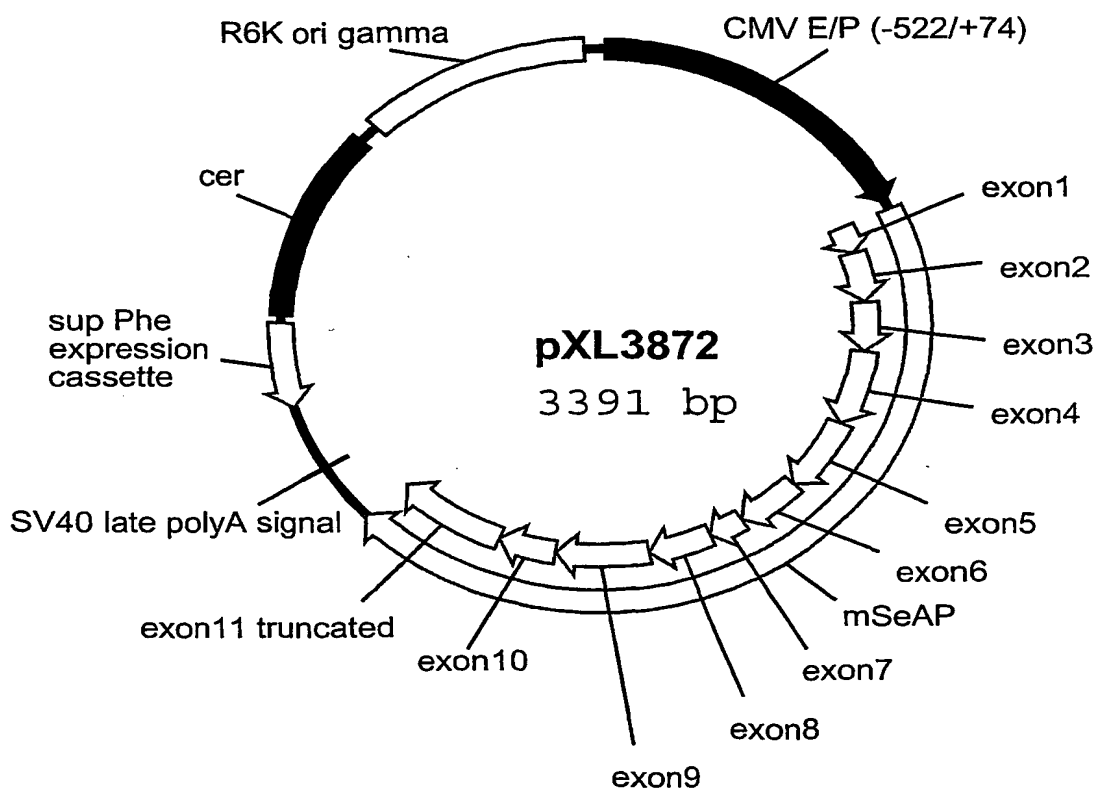
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Figure 2A

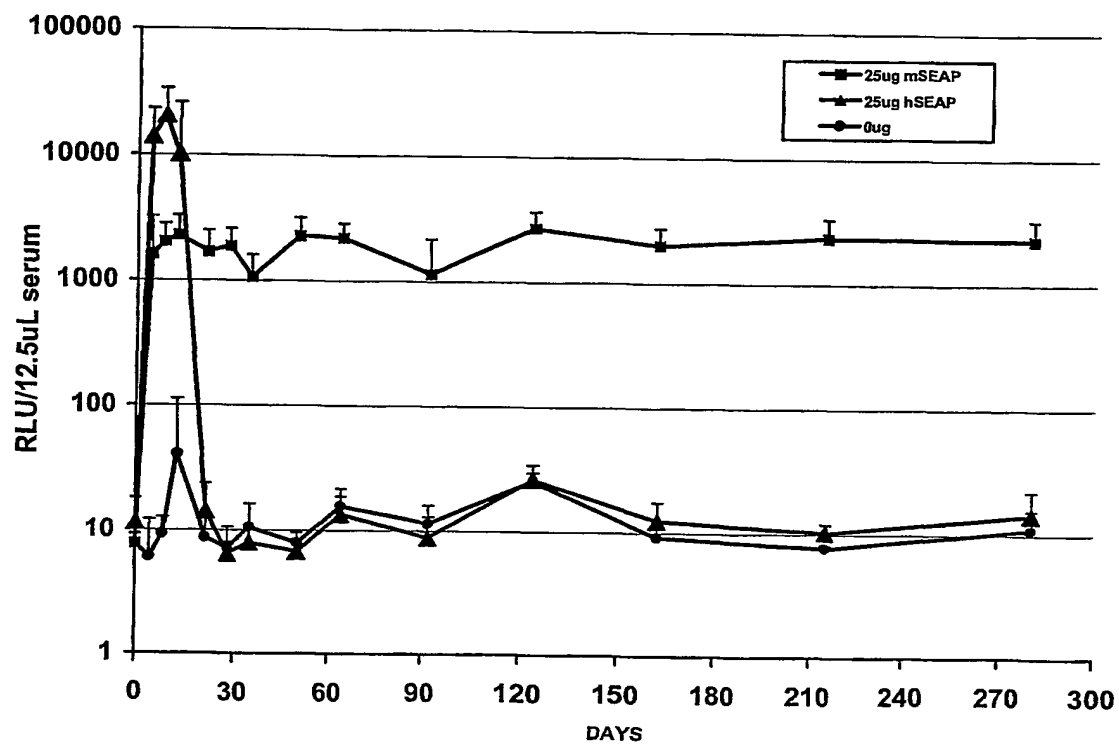
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Figure 2B

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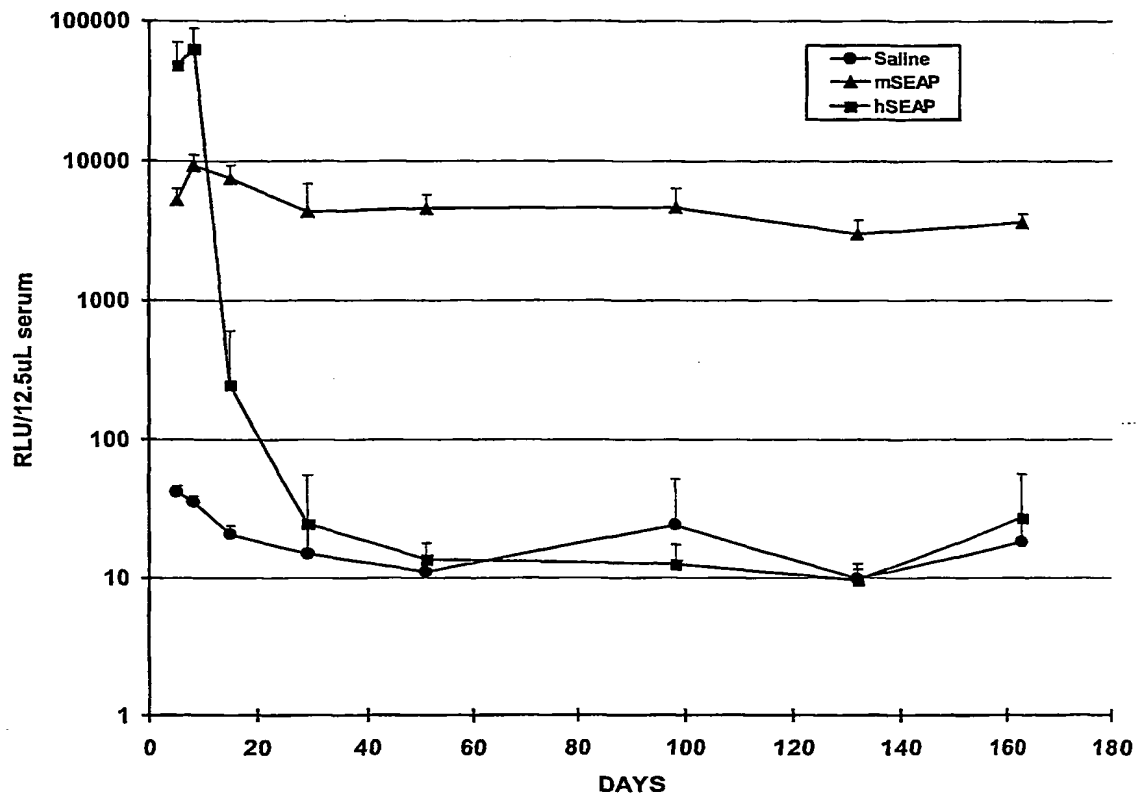
Figure 2C

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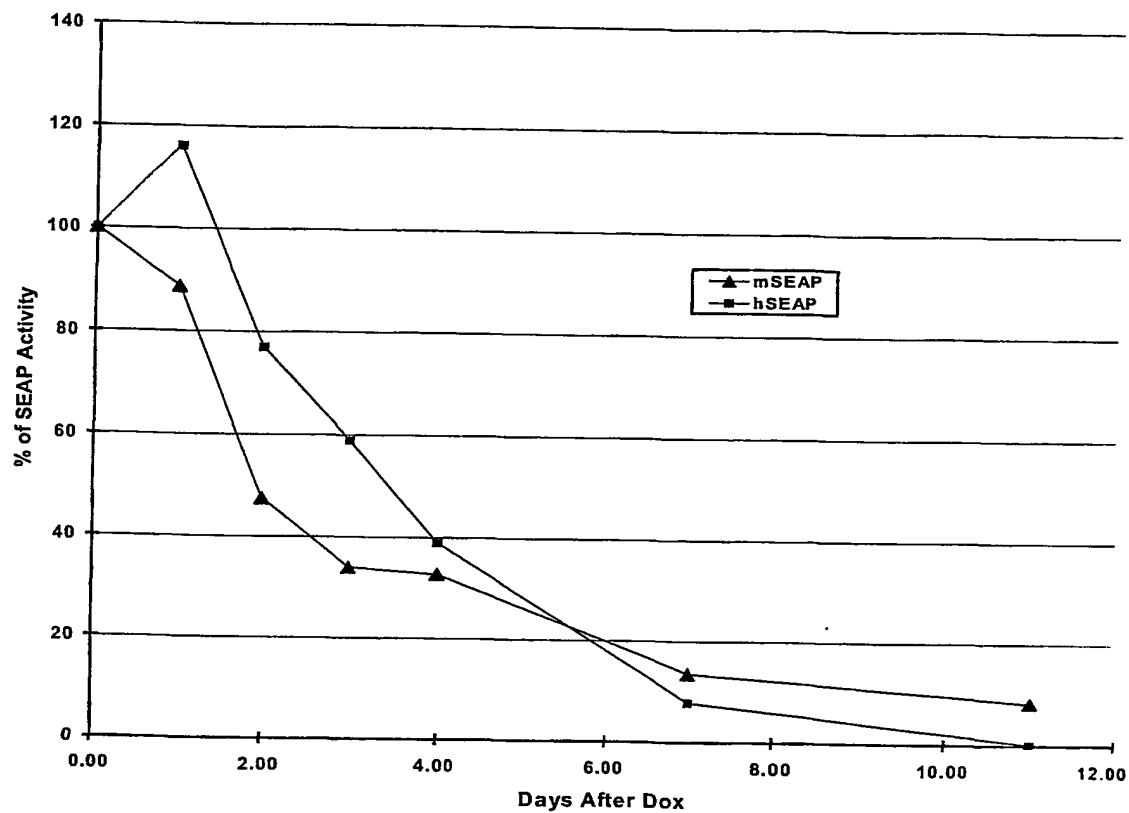
Figure 3

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Figure 4



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Figure 5



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Figure 6

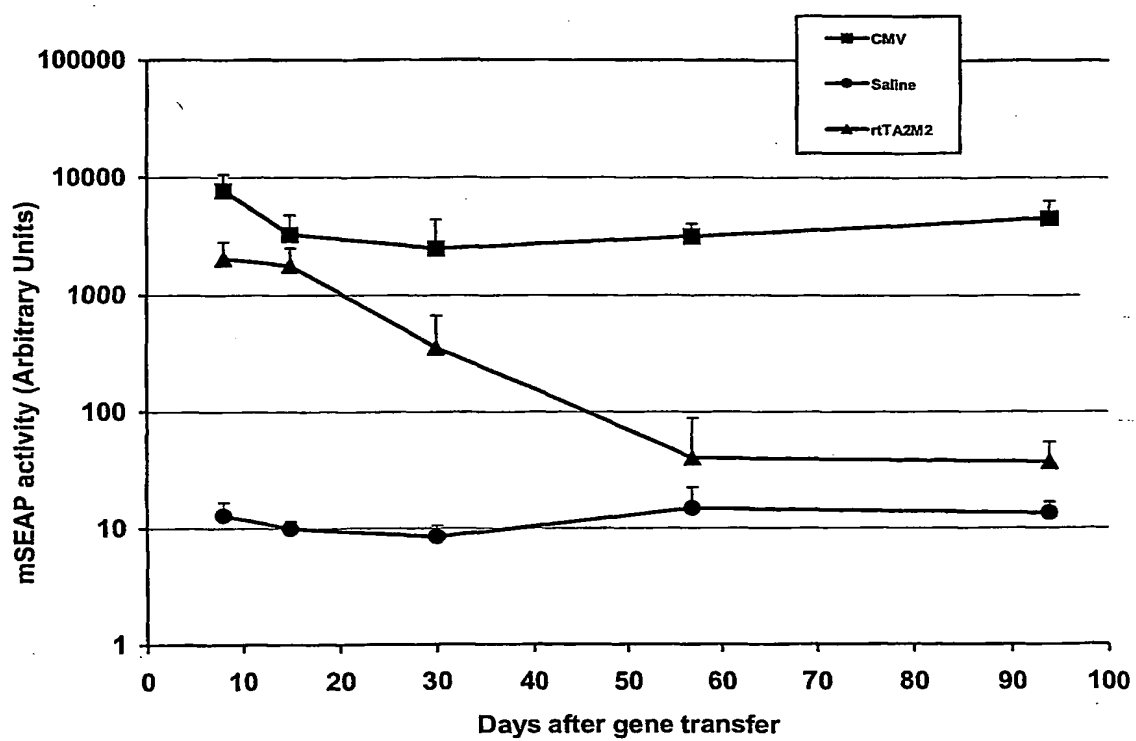


Figure 7

mSEAP- substitution sites

(1) MWGACLLLLGLSLQLCPSIIIPVEENPAFWNRKAAEALDAAKLKPIQTSAKNLVILMGD
 (61) GMGVSTVTATRIILKGQQQGHLPETQLAMDRFFPHMALSKTYNTDKQIPDSAGTGTAFLCG
 (121) VKTNMKVIGLSAAARFNQCNTTWGNEVVSVMHRAKKAGKSGVVTTTSVQHASPAGTYAH
 (181) TVNRGWYSDAQMPASALQDQCKDISTQLISNMDIDVILGGGRKFMFPKGTPDQEYPTDTK
 (241) QAGTRLDGRNLVQEWLAKHQGARYVWNRSELIQASLNRSVTHLMGLFEPNDMKYEIHRDP
 (301) AQDPSLAEMTEVAVRMLSRNPKGFLFVEGGRIDHGHHEITVAYRALTEAVMFD^{SAVD}KAD
 (361) KLTSEQDTMILVTADHSHVFSFGGYTQRGASIFGLAPFKAEDGKSFTSILYGNPGPYKLH
 (421) NGARADVTEESSNPTYQQQAAVPLSSETHSGEDVAIFARGPQAHLVHGVQEQNYIAHVM
 (481) AFAACLEPYTDCGLASPAGQSSAVSPG

SEQUENCE LISTING

<110> Thuillier, Vincent
Wang, Manping
Orsini, Cecile

<120> Engineered Secreted Alkaline Phosphatase (SEAP) Reporter Genes and Polypeptides

<130> GCUS001/0002

<150> 60/293,186

<151> 2001-05-25

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<170> PatentIn version 3.1

<210> 1

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<213> Artificial

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<223> Recombinant AP-activity polypeptide

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20 25 30

Lys Asn Leu Val Ile Leu Met Gly Asp Gly Met Gly Val Ser Thr Val
35 40 45

Thr Ala Thr Arg Ile Leu Lys Gly Gln Gln Gln Gly His Leu Gly Pro
50 55 60

Glu Thr Gln Leu Ala Met Asp Arg Phe Pro His Met Ala Leu Ser Lys
65 70 75 80

Thr Tyr Asn Thr Asp Lys Gln Ile Pro Asp Ser Ala Gly Thr Gly Thr
85 90 95

Ala Phe Leu Cys Gly Val Lys Thr Asn Met Lys Val Ile Gly Leu Ser
100 105 110

Ala Ala Ala Arg Phe Asn Gln Cys Asn Thr Thr Trp Gly Asn Glu Val
115 120 125

Val Ser Val Met His Arg Ala Lys Lys Ala Gly Lys Ser Val Gly Val
 130 135 140

Val Thr Thr Thr Ser Val Gln His Ala Ser Pro Ala Gly Thr Tyr Ala
 145 150 155 160

His Thr Val Asn Arg Gly Trp Tyr Ser Asp Ala Gln Met Pro Ala Ser
 165 170 175

Ala Leu Gln Asp Gly Cys Lys Asp Ile Ser Thr Gln Leu Ile Ser Asn
 180 185 190

Met Asp Ile Asp Val Ile Leu Gly Gly Gly Arg Lys Phe Met Phe Pro
 195 200 205

Lys Gly Thr Pro Asp Gln Glu Tyr Pro Thr Asp Thr Lys Gln Ala Gly
 210 215 220

Thr Arg Leu Asp Gly Arg Asn Leu Val Gln Glu Trp Leu Ala Lys His
 225 230 235 240

Gln Gly Ala Arg Tyr Val Trp Asn Arg Ser Glu Leu Ile Gln Ala Ser
 245 250 255

Leu Asn Arg Ser Val Thr His Leu Met Gly Leu Phe Glu Pro Asn Asp
 260 265 270

Met Lys Tyr Glu Ile His Arg Asp Pro Ala Gln Asp Pro Ser Leu Ala
 275 280 285

Glu Met Thr Glu Val Ala Val Arg Met Leu Ser Arg Asn Pro Lys Gly
 290 295 300

Phe Tyr Leu Phe Val Glu Gly Gly Arg Ile Asp His Gly His His Glu
 305 310 315 320

Thr Val Ala Tyr Arg Ala Leu Thr Glu Ala Val Met Phe Asp Ser Ala
 325 330 335

Val Asp Lys Ala Asp Lys Leu Thr Ser Glu Gln Asp Thr Met Ile Leu
 340 345 350

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Val Thr Ala Asp His Ser His Val Phe Ser Phe Gly Gly Tyr Thr Gln
 355 360 365

Arg Gly Ala Ser Ile Phe Gly Leu Ala Pro Phe Lys Ala Glu Asp Gly
 370 375 380

Lys Ser Phe Thr Ser Ile Leu Tyr Gly Asn Gly Pro Gly Tyr Lys Leu
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His Asn Gly Ala Arg Ala Asp Val Thr Glu Glu Glu Ser Ser Asn Pro
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Thr Tyr Gln Gln Gln Ala Ala Val Pro Leu Ser Ser Glu Thr His Ser
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Gly Glu Asp Val Ala Ile Phe Ala Arg Gly Pro Gln Ala His Leu Val
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His Gly Val Gln Glu Gln Asn Tyr Ile Ala His Val Met Ala Phe Ala
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Lys Ala Ala Glu Ala Leu Asp Ala Ala Lys Lys Leu Lys Pro Ile Gln
 35 40 45

Thr Ser Ala Lys Asn Leu Val Ile Leu Met Gly Asp Gly Met Gly Val
50 55 60

Ser Thr Val Thr Ala Thr Arg Ile Leu Lys Gly Gln Gln Gln Gly His
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Leu Gly Pro Glu Thr Gln Leu Ala Met Asp Arg Phe Pro His Met Ala
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Leu Ser Lys Thr Tyr Asn Thr Asp Lys Gln Ile Pro Asp Ser Ala Gly
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Thr Gly Thr Ala Phe Leu Cys Gly Val Lys Thr Asn Met Lys Val Ile
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Gly Leu Ser Ala Ala Ala Arg Phe Asn Gln Cys Asn Thr Thr Trp Gly
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Gln Ala Gly Thr Arg Leu Asp Gly Arg Asn Leu Val Gln Glu Trp Leu
245 250 255

Ala Lys His Gln Gly Ala Arg Tyr Val Trp Asn Arg Ser Glu Leu Ile
260 265 270

Gln Ala Ser Leu Asn Arg Ser Val Thr His Leu Met Gly Leu Phe Glu
275 280 285

Pro Asn Asp Met Lys Tyr Glu Ile His Arg Asp Pro Ala Gln Asp Pro
290 295 300

Ser Leu Ala Glu Met Thr Glu Val Ala Val Arg Met Leu Ser Arg Asn
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Pro Lys Gly Phe Tyr Leu Phe Val Glu Gly Gly Arg Ile Asp His Gly
325 330 335

His His Glu Thr Val Ala Tyr Arg Ala Leu Thr Glu Ala Val Met Phe
340 345 350

Asp Ser Ala Val Asp Lys Ala Asp Lys Leu Thr Ser Glu Gln Asp Thr
355 360 365

Met Ile Leu Val Thr Ala Asp His Ser His Val Phe Ser Phe Gly Gly
370 375 380

Tyr Thr Gln Arg Gly Ala Ser Ile Phe Gly Leu Ala Pro Phe Lys Ala
385 390 395 400

Glu Asp Gly Lys Ser Phe Thr Ser Ile Leu Tyr Gly Asn Gly Pro Gly
405 410 415

Tyr Lys Leu His Asn Gly Ala Arg Ala Asp Val Thr Glu Glu Glu Ser
420 425 430

Ser Asn Pro Thr Tyr Gln Gln Gln Ala Ala Val Pro Leu Ser Ser Glu
435 440 445

Thr His Ser Gly Glu Asp Val Ala Ile Phe Ala Arg Gly Pro Gln Ala
450 455 460

His Leu Val His Gly Val Gln Glu Gln Asn Tyr Ile Ala His Val Met
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Pro Ser Val

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<223> Human Preproinsulin signal peptide (GenBank GI:69300)

<220>

<221> PROTEIN

<222> (1) .. (24)

<223>

<400> 9

Met Ala Leu Trp Met Arg Leu Leu Pro Leu Leu Ala Leu Leu Ala Leu
1 5 10 15

Trp Gly Pro Asp Pro Ala Ala Ala
20

<210> 10

<211> 24

<212> PRT

<213> Artificial

<220>
<223> Mouse Preproinsulin (SwissProt P01325)

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<222>    (1) .. (24)
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Met Ala Leu Leu Val His Phe Leu Pro Leu Leu Ala Leu Leu Ala Leu
1 5 10 15

Trp Glu Pro Lys Pro Thr Gln Ala
20

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<210> 11
<211> 20
<212> PRT
<213> Artificial
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<223> Immunoglobulin gamma 2a-chain V-region signal peptide (GenBank GI:12750776)

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<222>  (1) .. (20)
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<400> 11

Met Glu Arg His Trp Ile Phe Leu Phe Leu Phe Ser Val Thr Ala Gly
1 5 10 15

Val His Ser Gln
20

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<210> 12
<211> 20
<212> PRT
<213> Artificial
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<220>
<223> Rat interleukin 2 precursor signal peptide (SwissProt P17108)
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<221>  PEPTIDE
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<223>  Rat interleukin 2 precursor signal peptide (swissprot P17108)
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<220>

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<221> PEPTIDE
<222> (1) .. (20)
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<400> 12

Met Tyr Ser Met Gln Leu Ala Ser Cys Val Ala Leu Thr Leu Val Leu
1 5 10 15

Leu Val Asn Ser
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<210> 13
<211> 529
<212> PRT
<213> Artificial

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<223> Full length mouse EAP polypeptide. (GenBank GI:192977)

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Met Trp Gly Ala Cys Leu Leu Leu Leu Gly Leu Ser Leu Gln Val Cys
1 5 10 15

Pro Ser Val Ile Pro Val Glu Glu Glu Asn Pro Ala Phe Trp Asn Arg
20 25 30

Lys Ala Ala Glu Ala Leu Asp Ala Ala Lys Lys Leu Lys Pro Ile Gln
35 40 45

Thr Ser Ala Lys Asn Leu Val Ile Leu Met Gly Asp Gly Met Gly Val
50 55 60

Ser Thr Val Thr Ala Thr Arg Ile Leu Lys Gly Gln Gln Gln Gly Leu
65 70 75 80

Ser Gly Pro Glu Thr Gln Leu Ala Met Asp Arg Phe Pro His Met Ala
85 90 95

Leu Ser Lys Thr Tyr Asn Thr Asp Lys Gln Ile Pro Asp Ser Ala Gly
100 105 110

Thr Gly Thr Ala Phe Leu Cys Gly Val Lys Thr Asn Met Lys Val Ile
115 120 125

Gly Leu Ser Ala Ala Ala Arg Phe Asn Gln Cys Asn Thr Thr Trp Gly
130 135 140

Asn Glu Val Val Ser Val Met His Arg Ala Lys Lys Ala Gly Lys Ser
 145 150 155 160

Val Gly Val Val Thr Thr Thr Ser Val Gln His Ala Ser Pro Ala Gly
 165 170 175

Thr Tyr Ala His Thr Val Asn Arg Gly Trp Tyr Ser Asp Ala Gln Met
 180 185 190

Pro Ala Ser Ala Leu Gln Asp Gly Cys Lys Asp Ile Ser Thr Gln Leu
 195 200 205

Ile Ser Asn Met Asp Ile Asp Val Ile Leu Gly Gly Gly Arg Lys Phe
 210 215 220

Met Phe Pro Lys Gly Thr Pro Asp Gln Glu Tyr Pro Thr Asp Thr Lys
 225 230 235 240

Gln Ala Gly Thr Arg Leu Asp Gly Arg Asn Leu Val Gln Glu Trp Leu
 245 250 255

Ala Lys His Gln Gly Ala Arg Tyr Val Trp Asn Arg Ser Glu Leu Ile
 260 265 270

Gln Ala Ser Leu Asn Arg Ser Val Thr His Leu Met Gly Leu Phe Glu
 275 280 285

Pro Asn Asp Met Lys Tyr Glu Ile His Arg Asp Pro Ala Gln Asp Pro
 290 295 300

Ser Leu Ala Glu Met Thr Glu Val Ala Val Arg Met Leu Ser Arg Asn
 305 310 315 320

Pro Lys Gly Phe Tyr Leu Phe Val Glu Gly Gly Arg Ile Asp His Gly
 325 330 335

His His Glu Thr Val Ala Tyr Arg Ala Leu Thr Glu Ala Val Met Phe
 340 345 350

Asp Ser Ala Val Asp Lys Ala Asp Ile Arg Thr Ser Glu Gln Asp Thr
 355 360 365

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Met Ile Leu Val Thr Ala Asp His Ser His Val Phe Ser Phe Gly Gly
370 375 380

Tyr Thr Gln Arg Gly Ala Ser Ile Phe Gly Leu Ala Pro Phe Lys Ala
385 390 395 400

Glu Asp Gly Lys Ser Phe Thr Ser Ile Leu Tyr Gly Asn Gly Pro Gly
405 410 415

Tyr Lys Leu His Asn Gly Ala Arg Ala Asp Val Thr Glu Glu Glu Ser
420 425 430

Ser Asn Pro Thr Tyr Gln Gln Gln Ala Cys Val Pro Leu Ser Ser Glu
435 440 445

Thr His Ser Gly Glu Asp Val Ala Ile Phe Ala Arg Gly Pro Gln Ala
450 455 460

His Leu Val His Gly Val Gln Glu Gln Asn Tyr Ile Ala His Val Met
465 470 475 480

Ala Phe Ala Ala Cys Leu Glu Pro Tyr Thr Asp Cys Gly Leu Ala Ser
485 490 495

Pro Ala Gly Gln Ser Ser Ala Val Ser Pro Gly Tyr Met Ser Thr Leu
500 505 510

Leu Cys Leu Leu Ala Gly Lys Met Leu Met Leu Met Ala Ala Ala Glu
515 520 525

Pro

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